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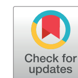
Crystal structure of EGFR T790M/C797S/V948R in complex with EAI045



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A B S T R A C T

Lung cancer is the leading cause of cancer deaths. Epidermal growth factor receptor (EGFR) kinase domain mutations are a common cause of non-small cell lung cancers (NSCLCs), a major subtype of lung cancers. Patients harboring most of these mutations respond well to the anti-EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib initially, but soon develop resistance to them in about half of the cases due to the emergence of the gatekeeper mutation T790M. The third-generation TKIs such as AZD9291, HM61713, CO-1686 and WZ4002 can overcome T790M through covalent binding to the EGFR kinase through Cys 797, but ultimately lose their efficacy upon emergence of the C797S mutation that abolishes the covalent bonding. Therefore to develop new TKIs to overcome EGFR drug-resistant mutants harboring T790M/C797S is urgently demanded. EAI001 and EAI045 are a new type of EGFR TKIs that bind to EGFR reversibly and not relying on Cys 797. EAI045 in combination with cetuximab is effective in mouse models of lung cancer driven by EGFR L858R/T790M and L858R/T790M/C797S. Here we report the crystal structure of EGFR T790M/C797S/V948R in complex with EAI045, and compare it to EGFR T790M/V948R in complex with EAI001. The complex structure reveals why EAI045 binds tighter to EGFR than does EAI001, and why EAI001 and EAI045 prefer binding to EGFR T790M. The knowledge may facilitate future drug development studies targeting this very important cancer target.

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1. Introduction

Lung cancer accounts for nearly one third of all cancer deaths every year [1]. The most frequently seen lung cancer histologic subtype is non-small cell lung cancer (NSCLC), which accounts for about 85% of all lung cancer cases [2,3]. Activating mutations in the EGFR kinase domain, typically including the exon 18 single site mutation G719X, exon 19 deletion mutations (designated "Del-19s", among which the most common one is delE746-A750), exon 20 insertions and exon 21 single site mutation L858R have been identified as the common causes of NSCLCs, which are found in about 10–15% Caucasian patients and 30–40% Asian patients, more frequently in women and never or light cigarette smokers [4,5]. Among these EGFR kinase mutations the most commonly seen types are Del-19 and L858R. Patients harboring G719X, L858R and Del-19s usually respond very well to the so-called "fist-generation" anti-EGFR TKIs gefitinib and erlotinib [4–7]. Unfortunately the duration of the efficacy of these agents is limited due to the emergence of drug-resistance. In about half of the relapsed cases, resistance to gefitinib/erlotinib is caused by a secondary mutation in EGFR kinase that results in the substitution of Threonine to Methione at residue 790 (T790M, the "gatekeeper" mutation) [8,9].

WZ4002, AZD9291, CO-1686 and HM61713 are the newly developed "third-generation" EGFR TKIs that efficiently overcome the EGFR T790M drug-resistance mutation while sparing the wild-type EGFR [10–13] (Fig. 1). CO-1686 and AZD9291 exhibited excellent clinical efficacy in NSCLC patients harboring EGFR T790M with more than 50% response rates and less skin and gastrointestinal toxicities than those typically observed for the first generation EGFR TKIs [14,15]. Recently, AZD9291 was approved by FDA for the treatment of patients with metastatic EGFR T790M mutation-positive NSCLC who have progressed on or after EGFR TKI therapy [16]. Unfortunately, resistance to the third-generation TKIs may

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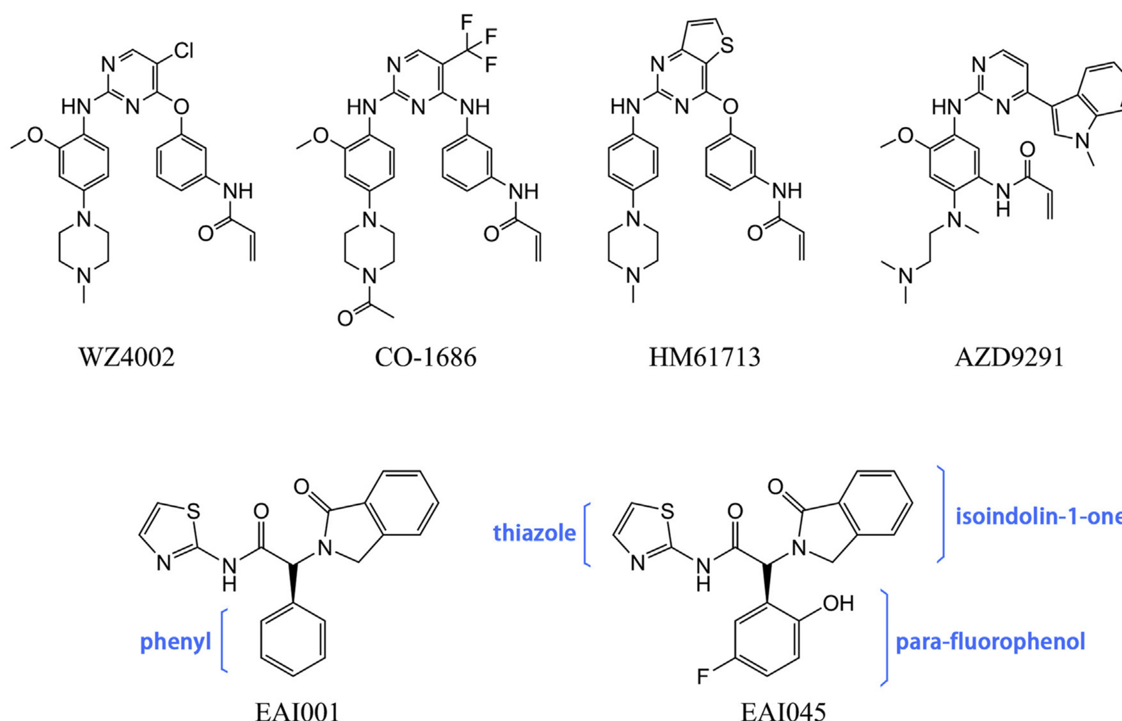

Fig. 1. Chemical structures of the compounds discussed in this report.

emerge during the treatment. Ercan *et al*. performed an N-ethyl-N-nitrosourea (ENU) mutagenesis screen in EGFR-mutant (sensitizing alone or with concurrent EGFR T790M) Ba/F3 cells and selected WZ4002-resistant clones. In this study three new mutations, C797S, L718Q and L844V were found to confer resistance to WZ4002. It was found that C797S is resistant to all three agents, *i.e.*, WZ4002, CO-1686 and AZD9291, while L718Q and L844V mutations are resistant to WZ4002 and CO-1686, but not AZD9291 [17]. More recently, C797S was confirmed to be an acquired drug-resistant mutation in non-small cell lung cancer patients harboring EGFR T790M and receiving AZD9291 and HM61713 treatment [18,19]. New agents to overcome the EGFR mutations L858 R/T790M and Del-19/T790M with concomitant C797S mutation are therefore highly demanded.

WZ4002, CO-1686, AZD9291 and HM61713 share the same anilinopyrimidine core scaffold and an acrylamide warhead. They all depend on covalent linkage through the thiol group of Cys 797 side-chain to form a covalent linkage with EGFR [10–13,20]. Substitution of thiol by hydroxyl on residue 797 (the C797S mutation) would therefore abolish the covalent binding of these inhibitors, which at least partly explains the mechanism of resistance to these agents. The next generation drugs to overcome EGFR T790M/C797S (in the presence of a primary oncogenic mutation such as L858R or Del-19) still need to compete with ATP without losing specificity to the mutant EGFR, which is very difficult because ATP binds to EGFR T790M tightly [21].

A workaround is to inhibit the EGFR kinase allosterically, *i.e.*, to inhibit EGFR kinase using small compounds not binding in the ATP-binding pocket and therefore not competing with ATP. Recently, we reported the first EGFR allosteric inhibitor (EAI) EAI045. This compound in combination with the antibody cetuximab can inhibit EGFR L858 R/T790M and L858 R/T790M/C797S very well both *in vitro* and in an animal model [22]. EAI045 was developed on the basis of the hit compound EAI001 that was identified in a high-throughput screening. EAI001 inhibits L858 R/T790M with an IC_{50} of about 0.024 μM, while EAI045 binds to the same target with an IC_{50} of about 0.003 μM. However, due to lack of an EGFR/EAI045 complex crystal structure, it remains elusive why EAI045 binds to EGFR T790M with higher affinity than EAI001. Here we report the crystal structure of EGFR T790M/C797S/V948R in complex with EAI045. This structure illustrate the binding details of this compound to EGFR T790M/C797S, and explains why it is more potent than EAI001.

2. Materials and methods

2.1. Cloning, expression, and purification of EGFR T790M/C797S/V948R

DNA encoding residues 675–1022 of the human EGFR was PCR-cloned from the full-length cDNA of human EGFR and inserted into the pFastBac™ HTA vector (Invitrogen). The T790M, C797S and V948R mutations were then introduced in the construct one by one by site-directed mutagenesis using the QuikChange method (Stratagene). The baculovirus to express this protein in insect cells was prepared using the Bac-to-Bac system according to the official protocol (Invitrogen). A 6×His tag was added to the C-terminus of the EGFR protein.

The EGFR proteins were expressed in sf9 insect cells. After harvesting, the cell were broken by sonication in lysis buffer (30 mM Tris, 150 mM NaCl, 3 mM KCl, 10% glycerol, 1 mM PMSF, 1 mM TCEP, 1x protease inhibitor mixture (Complete EDTA-free, Roche), 20 mM Imidazole, pH7.4). Cell lysate was obtained by centrifugation at 20,000 rpm for 1 hour at 4 °C and then applied to the Chelating Sepharose Beads (GE Healthcare, Cat. 17-0575-02) charged with Ni^{2+}. The beads were thoroughly washed with wash buffer (20 mM Tris, 500 mM NaCl, 1% glycerol, 0.5 mM TCEP, 30 mM Imidazole, pH 8.0), and then the protein was eluted with elution buffer (20 mM Tris, 500 mM NaCl, 1% glycerol, 1 mM TCEP, 300 mM Imidazole, pH 8.0). The eluent was concentrated and then applied

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